MYC regulation of CHK1 and CHK2 promotes radioresistance in a stem cell-like population of nasopharyngeal carcinoma cells

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Abstract
Radiotherapy is the most successful nonsurgical treatment for nasopharyngeal carcinoma (NPC). Despite this, the prognosis remains poor. Although NPCs initially respond well to a full course of radiation, recurrence is frequent. The cancer stem cell (CSC) hypothesis provides a framework for explaining the discrepancy between the response of NPC to therapy and the poor survival rate. In this study, a stem cell-like subpopulation (PKH26+) was identified in NPC cell lines using a label retention technique. PKH26+ cells were enriched for clonogenicity, sphere-formation, side-population cells, and resistance to radiotherapy. Using genomic approaches, we show that the proto-oncogene c-MYC (MYC) regulates radio-tolerance through transcriptional activation of CHK1 (CHEK1) and CHK2 (CHEK2) checkpoint kinases through direct binding to the CHK1 and CHK2 promoters. Overexpression of c-MYC in the PKH26+ subpopulation leads to increased expression of CHK1 and CHK2 and subsequent activation of the DNA damage checkpoint response, resulting in radioresistance. Furthermore, loss of CHK1 and CHK2 expression reverses radioresistance in PKH26+ (c-MYC high expression) cells in vitro and in vivo. This study elucidates the role of the c-MYC-CHK1/CHK2 axis in regulating DNA damage checkpoint responses and stem cell characteristics in the PKH26+ subpopulation. Furthermore, these data reveal a potential therapeutic application in reversal of radioresistance through inhibition of the c-MYC-CHK1/CHK2 pathway.
Introduction

Nasopharyngeal carcinoma (NPC) is a rare form of epithelial cancer that occurs in most parts of the world. However, its occurrence is particularly high in Southeast Asia and southern China where its incidence rate is approximately 25 to 50 cases per 100,000 individuals 25-fold higher than that in western countries (1). Surgical approaches to treat NPC are limited by the inaccessibility of the anatomic location. However, NPC is sensitive to radiation and, therefore, treatments primarily rely on radiotherapy. However, approximately 30% of patients presenting with localized tumors develop recurrent disease, and 30% to 60% of patients with metastatic NPC die within 5 years of diagnosis (2).

In recent years, the concept of cancer stem cell (CSC) has been proposed, which is defined as a cell within a tumor that possesses the capacity to self-renew and to generate the heterogeneous lineages of cancer cells that comprise the tumor (3). This concept has been extensively investigated following the identification of CSCs in diverse cancers including breast, brain, lung, and liver (4-7). Analyses are based on the exploitation of CSC surface markers, elevated levels of aldehyde dehydrogenase (ALDH1+), and enhanced PKH26 dye-retaining capacity (8, 9). This has raised the prospect of human CSC isolation. Recent data suggest that CSCs are more resistant to chemo- and radiotherapy than non-stem cells (10). These key properties enable CSCs to initiate tumors and promote cancer progression and may account for the failure of current therapies to eradicate malignant tumors. A recent study demonstrated that
CSCs contribute to radioresistance through preferential activation of the CHK1 and CHK2 checkpoint response and an increase in DNA repair capacity (11).

The c-MYC oncoprotein is a well-characterized transcription factor, and deregulation of c-MYC contributes to the genesis of most human tumors (12). As a result, c-MYC is considered to play an important role in carcinogenesis and tumor progression due to its influence on all basic cellular processes (13). Myc can activate cyclin E/Cdk2 in quiescent fibroblasts (14) by inhibiting the cdk2 inhibitor p27 (15). Myc was reported to cooperate with p53 and Pten in the regulation of normal and malignant stem/progenitor cell differentiation, self-renewal and tumorigenic potential (16). Previous research has also indicated that c-MYC network accounts for similarities between embryonic stem and cancer cell transcription programs (17).

The aim of this study is to determine the capacity of PKH26 dye retention to successfully identify slow cycling PKH26 + cells (18). We determined that these cells were radioresistant. It has also been shown that c-MYC can directly regulate CHK1/2. Here, we explore the role of c-MYC in the radioresistance of nasopharyngeal carcinoma, and the molecular mechanisms of DNA damage repair that are implicated in this process.

Materials and Methods

Cells and culture conditions

NPC cell lines (CNE1, CNE2, SUNE1, HONE1) and NSCLC cell line (A549) were available from the Cancer Institute of Southern Medical University (Guangzhou, China). The authenticity of cell lines in our study have verified with the DNA finger
printing method. All cell lines used in this study were maintained in RPMI 1640 medium (Invitrogen) with 10% fetal bovine serum (FBS) (HyClone), 100 U/ml penicillin, 100 mg/ml streptomycin (Invitrogen), and incubated at 5% CO₂ at 37°C. 

**PKH26 labeling of cells and sorting of cell populations**

CNE1 and CNE2 cells were labeled using PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich) prior to cell culture, according to the instructions provided by the manufacturer. PKH26 is a lipophilic marker that intercalates into the membranes of viable cells (18) and is not transferred between cells. Labeled CNE2 cells were cultured for about 4 weeks. PKH26+ and PKH26- fractions were identified by fluorescence activated cell sorting (FACS). Freshly PKH26-labeled cells and unlabeled cells were used as positive and negative controls, respectively. Cells were sorted for subsequent analyses. After sorting, PKH26+ cells were cultured in serum-free stem cell medium (MEBM; Clonetics division of Cambrex BioScience) to maintain stem cell characteristics. PKH26- and unsorted cells were cultured in RPMI-1640 medium.

**Cell cycle analysis by FACS**

Flow cytometry was performed as previously described (19). Briefly, cells were trypsinized, washed with PBS, resuspended in 70% ethanol, and stored at -20°C overnight. Cells were subsequently centrifuged, washed in PBS, resuspended in 450 μl PBS and 10 μl 10 μg/ml DNase-free RNase (Roche), and incubated at 37°C for 45 min. Following RNase treatment, 50 μl of propidium iodide (Boehringer Mannheim Corp.) was added, and cells were incubated at room temperature for 10 min protected
from light. Cell aggregates were removed by filtration prior to analysis. Cell cycle analysis was carried out using the BD FACSDiva™. The population of cells in each of the G1, S, M, and G2 phases was determined for at least 250,000 cells with doublet discrimination. Analysis of cell cycle position was performed using the BD FACSDiva software.

**Tumor spheroid assay**

Spheroid-forming assays were performed as previously described (20). In brief, single cells were plated in 6-well ultralow attachment plates (Corning Inc., Corning, NY, USA) at a density of 1,000 cells/ml in tumorsphere culture medium (DMEM+F12 supplemented with 1% N2 Supplement (Invitrogen), 2% B27 Supplement (Invitrogen), 20 ng/ml human platelet growth factor (Sigma-Aldrich), 100 ng/ml epidermal growth factor (Invitrogen), and 1% antibiotic-antimycotic (Invitrogen)) at 37°C in a humidified atmosphere of 95% air and 5% CO2. Cultures were fed weekly and passaged every two weeks. When passaged, tumorspheres were harvested. Spheroids were dissociated with Accutase (Innovative Cell Technologies, Inc.).

**Antibodies, Western blot, and immunofluorescence analysis**

Western blot and immunofluorescence analyses were performed according to standard protocols (21) using the following antibodies: mouse anti-human cyclin D1, cyclin A, and cyclin B (1:300; Santa Cruz), mouse anti-human ABCG2, CD-44, CHK1, CHK2, pCHK1, pCHK2 GAPDH (1:1000; Cell Signaling), γ-H2AX (1:1000; Abcam). Peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:500; Cell Signaling) was used as the secondary detection antibody. Quantification of blots was
performed using Image J software (National Institutes of Health). Chamber slides were analyzed using a Nikon Eclipse fluorescence microscope.

**Xenograft Experiments**

Animal studies were conducted in strict accordance with the principles and procedures approved by the Committee on the Ethics of Animal Experiments of Southern Medical University. Nude mice (BALB/C nu/nu) were fed autoclaved water and laboratory rodent chow. Freshly sorted PKH26+ and PKH26- cells suspended in 200 μL PBS were inoculated into the flanks of 6- to 7-week-old nude female mice on the afternoon of the day of sorting. The mice were monitored twice weekly for palpable tumor formation and euthanized 4 weeks after transplantation to assess tumor formation. Tumors were measured using a Vernier caliper, weighed, and photographed. A portion of the s.c. tumor tissue was collected, fixed in 10% formaldehyde, and embedded in paraffin for H&E staining to assess tumor pathology.

To analyze the effect of IR and si-c-MYC as combination therapy, $5 \times 10^3$ PKH26+ derived from CNE2 cells were subcutaneously injected into athymic nude mice, and tumor volume was monitored every 5 days as calculated by the equation $V(\text{mm}^3) = \frac{a \times b^2}{2}$, where “a” is the largest diameter and “b” is the perpendicular diameter. When the tumors reached a size of 70 mm$^3$, mice were randomly distributed to six groups (four mice per group) and treated with IR (8Gy, 2×4Gy), si-c-MYC, or the combination of IR and si-c-MYC. There were four cycles (days 10, 24, 38, 52) of IR, and tumor volume was monitored at various times up to 55 days. In addition, cells obtained from the treated tumors were analyzed for the percentage of PKH26+ and
CD44+ by flow cytometry.

The Patients and Tissue Samples, Ethics Statement, Immunohistochemistry, c-MYCBS prediction and ChIP assay, EdU labeling, Luciferase assay Alkaline comet assay, Quantitative real-time (qRT)-PCR and Clonogenic survival assay are presented in the Supplemental Material and Methods.

**Statistical analysis**

Unless stated otherwise, all experiments were performed in triplicate. Data are expressed as the mean±SD of at least three independent experiments. The significance of differences between mean values was determined using Two-way ANOVA. *P*<0.05 was considered significant.

**Results**

**Cell sorting and PKH+ cell characterization**

After labeling nasopharyngeal carcinoma (NPC) cells with PKH26 and culturing for 30 days, we found that non-dividing or slowly dividing cells remain brightly labeled with membrane intercalating dyes (Fig.1A). We also found that these cells maintain adhesion and morphological integrity. After that, we used flow cytometry to isolate healthy PKH-negative (PKH-) and PHK-positive (PKH+) cells. Approximately 97.3% of NPC CNE1 cells and 94.7% of CNE2 cells were positive within the first week. However, at 2- and 4-weeks, respectively, there was a reduction in the number of PKH26 + cells (38.6% and 2.2% in CNE1; 47.9% and 2.8% in CNE2) (Fig.1B, Supplementary Figure 1B). The other two human NPC cell lines, SUNE-1 and HONE-1, also contained a small subpopulation of PKH+ cells, and, after four weeks
of labeling, they displayed a positive staining rate of 2.5% and 1.9%, respectively (data not shown). We sorted the top 2% from the total cell population in CNE1 and CNE2 cells after labeling with PKH26 for 4 weeks. Then we used FACS to determine cell cycle distribution. PKH26 dye has been previously used to trace cell divisions and identify slow cycling populations, such as long-term culture-initiating cells (22). As seen in Figure 1C, PKH+ cells showed higher fractions of cells in G1 phase and lower fractions of cells in S and G2-M phase. We next examined the expression of cell cycle-related proteins (Fig. 1D).

**PKH26+ cells are enriched for the stem cell-like subpopulation and resistant to radiotherapy**

The *in vivo* DNA-binding dye Hoechst 33342 was used to stain PKH26+ and PKH26-NPC cells. Simultaneous detection of the wavelengths of red and blue fluorescent dyes allowed selection of the two groups of specific subpopulations: the main group of highly fluorescent cells (main population, MP) and a low fluorescence side-population of cells (side-population, SP) (23). SP cells were sorted based on minimal nuclear staining by the fluorescent dye Hoechst 33342 due to cellular efflux (23). The proportion of SP cells was analyzed in CNE1 and CNE2 cells. Verapamil blocks efflux of Hoechst 33342 from SP cells and was used for detection of these cells. The data show that in sorted CNE1 cells, the PKH26+ population contained 32.1±5.2% SP cells, whereas the PKH26- population contained 0.5±0.3% SP cells. Using the same methods, we sorted CNE2 cells and found that the PKH26+ population contained 37.1±7.1% SP cells, and the PKH26- population contained only...
0.7±0.5% SP cells (Fig. 2A). Recent studies have reported that SP cells within NPC cells exhibit CSC characteristics (24). High expression of adenosine triphosphate binding cassette superfamily G member-2 transport protein (ABCG-2) has been closely related with SP cell phenotype (25). Western blot analysis of representative stem cell markers, ABCG2 and CD44, revealed expression of these proteins in all PKH26- and PKH26+ cells (Fig. 2B). CD44 has also been associated with cancer stem cell characteristics and has been specifically used as a marker for NPC stem cells (26). In order to verify that the PKH26+ cells also expressed CD44, we used flow cytometry and found that 85±3.4% and 93±1.4% of CNE1 and CNE2 PKH26+ cells, respectively, contained PKH26/CD44 double-positive cells (Supplementary Figure 1A). Clonogenic survival has been defined as a characteristic of radiation-induced cell proliferation and death. Therefore, the cloning properties of PKH26+ and PKH26- were evaluated. PKH26+ and PKH26- subpopulations of CNE1 and CNE2 cells were harvested and cultured for two weeks. PKH26+ cells gave a rise to significantly more colonies than PKH26- cells (Fig. 2C). Clonogenic survival of PKH26- and PKH26+ cells was determined after increasing doses of irradiation ($p<0.001$, Fig. 2D). The association of enhanced self-renewal properties with CSC properties was then investigated in tumor spheroid assays using single-cell suspensions derived from sorted PKH26+ and PKH26- cells. PKH26+ cells formed spheres that were significantly greater in number and larger than those formed by PKH26- cells (Fig. 2E). PKH26+ cells sorted from NPC cell line CNE2 and NSCLC cell line A549 were tested for the formation of xenografts in nude mice. We found that the PKH26+
cells grew faster (Fig.2F and Supplementary Figure 4A, 4B). Additionally, immunohistochemistry of Ki-67 detected increased expression in PKH26+ cells in our xenograft model (Fig. 2G).

**PKH26+ cells promote radioresistance through c-MYC overexpression**

DNA damage checkpoint responses play essential roles in cellular radiosensitivity (27-30). To determine the role of DNA damage checkpoint responses in PKH26+ subpopulation, we compared expression of early DNA damage checkpoint regulators in PKH26- and PKH26+ NPC cell subpopulations. We irradiated (8Gy) PKH26- and PKH26+ cells and measured protein expression of c-MYC, CHK1, pCHK1, CHKk2, and pCHK2 (Fig.3A). Irradiation increased levels of CHK1 and CHK2 phosphorylation in both PKH26- and PKH26+ cells. However, the activating phosphorylation of these checkpoint proteins was significantly higher in PKH26+ cells than in PKH26- cells, indicating that PKH26+ cells show greater checkpoint activation in response to DNA damage. In addition, we also found that PKH26+ cells expressed increased c-MYC compared to PKH26- cells. Immunofluorescence staining revealed that c-MYC, pCHK1, and pCHK2 were also localized to the nucleus in PKH26+ cells (Fig 3B). As mentioned above, c-MYC is highly expressed in PKH26+ NPC cells. Thus, we hypothesize that c-MYC is involved in DNA damage responses in NPC. To elucidate the relationship between c-MYC and the DNA damage response, we constructed a lentiviral vector of c-MYC to overexpress the transcription factor in the nasopharyngeal carcinoma cell lines CNE1 and CNE2. We found that DNA damage-related protein γ-H2AX expression
levels in the c-MYC over-expressing group is greatly reduced (Fig. 3C). Using comet assays, we found that the percentage of cells displaying DNA damage decreased by about 2.8-fold in both CNE1 and CNE2 cells over-expressing c-MYC (Fig 3D). EDU assay showed that following irradiation, cells over-expressing c-MYC showed improved proliferative capacity (Fig.3E). This result suggests that c-MYC over-expression can significantly reduce DNA damage and improve proliferative capacity in CNE1 and CNE2 cells after IR.

**c-MYC targets Chk1 and Chk2**

Experiments indicate that c-MYC may rapidly respond to IR in PKH26+ cells to allow increased DNA repair. In order to find whether c-MYC directly regulates *CHK1* and *CHK2*, we used the Gene Regulation website and Patch software (http://www.biobase-international.com/gene-regulation) to predict c-MYC binding sites within the promoter region of *CHK1* and *CHK2*. According to the Patch prediction, we found three potential c-MYCC binding sites (c-MYCBSs) within the 2000bp promoter upstream of the *CHK1* and *CHK2* genes, respectively (Fig. 4A). To validate direct association of c-MYC with *CHK1* and *CHK2* promoters, we performed chromatin immunoprecipitation (ChIP) analysis in NPC cells for all the putative c-MYCBSs (A-F) using a c-MYC antibody. The ChIP results suggest that c-MYC is most significantly bound to sites C and E within the *CHK1* and *CHK2* promoters (Fig. 4B). Knockdown of c-MYC diminished the amount of DNA c-MYCBSs C and E that could be immunoprecipitated with the c-MYC antibody (Fig. 4C), suggesting that c-MYC directly associates with these promoter regions. Next, we constructed a
luciferase reporter gene vector containing *CHK1* c-MYCBSs C and *CHK2* c-MYCBSs E to verify the predicted results. After overexpressed in NPC cells, c-MYC increases luciferase expression from the promoter reporter. To illustrate interaction between c-MYC and c-MYCBSs C and E from CHK1 and CHK2 promoters, we mutated the c-MYCBSs C and E in *CHK1* and *CHK2* luciferase reporter gene vectors. The results showed that mutated c-MYCBSs did not display increased luciferase activity after c-MYC overexpression, thus, confirming that c-MYC acts through *CHK1* c-MYCBSs C and *CHK2* c-MycBSs E (Fig. 4D). We also examined CHK1 and CHK2 protein and mRNA levels. The results showed that when overexpressed, c-MYC causes increased expression of CHK1 and CHK2 at both the protein and mRNA levels (Fig. 4 E and F). Together, these results suggest that c-MYC targets *CHK1* and *CHK2* directly by interaction with their promoter regions via sites c-MYCBSs C and E.

**PHK26*+ subpopulation promotes DNA repair capacity and stemness by activation of c-MYC expression**

Previous experiments have confirmed the following: high expression of c-MYC is consistent with CHK1 and CHK2 in PHK26*+ cells; PHK26*+ cells display greater DNA damage repair capacity and radiation resistance; and c-MYC over-expression significantly improves DNA damage repair after IR and increases stemness in NPC CNE1 and CNE2 cells. We hypothesize that c-MYC is also an essential factor in PHK26*+ cells to maintain their cancer stemness and DNA damage repair capacity.
following IR. To investigate the role of c-MYC in the DNA damage response and cancer stemness of PKH26+ subpopulation cells, we used lentivirus expressing shRNA specific to c-MYC (sh-c-MYC1 and sh-c-MYC2) (31), which constitutively expresses short hairpin RNAs (shRNAs) that specifically silence c-MYC expression (Fig 5A). Depletion of c-MYC levels in PKH26+ cells resulted in a decreased proportion of the stem cell-like side populations (SPs) in CNE1 PKH26+ cells (from 32.8±6.5% to 2.4±1.2%) and in CNE2 PKH26+ cells (from 35.6±7.1% to 2.0±3.3%) (Fig.5B). To further confirm our hypothesis, we sorted PKH26+ cells from CNE1 and CNE2 cell lines for tumor sphere formation assays. Inhibition of c-MYC by sh-c-MYC reduced the tumorsphere formation proportion in CNE1 PKH26+ cells (from ~128/1000 to ~42/1000 cells) and CNE2 PKH26+ cells (from ~122/1000 to ~38/1000 cells) (Fig.5C). To examine the effect of c-MYC in IR-induced DNA damage of PKH26+ subpopulaion cells in NPC, we performed alkaline comet assays. The percentage of cells with comet tails increased 3.1–3.7 times more rapidly in vector control-treated PKH26+ cells than in sh-c-MYC-treated PKH26+ cells in the NPC cell lines (Fig.5D). Moreover, EDU assays revealed that c-MYC inhibition reduces the proliferative capacity of PKH26+ cells after irradiation (Fig.5E). Inhibition of c-MYC in PKH26+ cells also decreases expression of CHK1, CHK2 (Supplementary Figure 3B), and increases the DNA damage related factor γ-H2AX expression after IR (Fig.5F.). Altogether, c-MYC plays an important role in PKH26+ cells to maintain cancer stemness and DNA damage repair capacity in response to IR.

**c-MYC Mediates DNA damage response and radioresistance in PKH26+ cells**
through modulating of CHK1/2

To further understand how c-MYC regulates the DNA damage response by modulating CHK1/2, we inhibited CHK1/2 expression in PKH26+ cells with shRNA. While depletion of CHK1/2 does not affect expression of c-MYC (Fig 6A Supplementary Figure 2A), it does impair DNA repair in c-MYC over-expressing PKH26+ cells after IR. The percentage of cells with comet tails increased 3–4.5 times more rapidly in vector control PKH26+ cells than in CHK1/2 shRNA infected PKH26+ and PKH26- cells (Fig 6B). To test whether the survival of PKH26+ cells is influenced by c-MYC after IR, we compared cell survival in vector control and sh-c-MYC lentivirus infected PKH26+ cells at different doses of IR. We found that inhibition of c-MYC reverses the IR resistance in PKH26+ (Fig. 6C). To further identify c-MYC mediated PKH26+ subpopulations that contribute to NPC radioresistance, we studied the radiosensitivity of PKH26- and PKH26+ tumor cell subpopulations. Colony formation assays confirmed that PKH26+ cells were more resistant to IR treatment than PKH26- cells. Additionally, inhibition of c-MYC resulted in sensitization of PKH26- and PKH26+ cells to IR (Fig. 6D). To further confirm that c-MYC regulates the DNA damage response through CHK1/2 in NPC, we compared survival of c-MYC knockdown cell with the co-treated (sh-c-MYC+ CHK1/2 overexpression) cells after IR using colony formation assays (Fig 6E). Significantly, CHK1, CHK2 and the DNA damage marker histone variant H2AX (γH2AX) (32), is dramatically induced by IR in the MYC knockdown PKH26+ cells, but not in control knockdown group or in the co-treated (sh-c-MYC+ CHK1/2
overexpression) PKH26+ cells (Fig. 6F). Moreover, CHK1/2 could reverse the effect of c-MYC in radioresistance in PKH26- cells (Supplementary Fig 2B, 2C). This result implies that c-MYC mediated DNA repair depends on activation of CHK1/2. Together, our results show that the c-MYC-CHK1/2 pathway plays an important role in mediating resistance of the PKH26+ subpopulation to IR.

c-MYC-CHK1/2 pathway is important for PKH26+ cell formation and maintenance of radioresistance in vivo

To verify the role of the c-MYC-CHK1/2 pathway for PKH26+ cell function in vivo, we performed xenograft experiments in which PKH26+ subpopulations derived from NPC cells were subcutaneously injected into nude mice. As expected, different IR treatments caused significant regression of the tumor, but relapse of the disease occurred after ~30 days. Treatment with si-c-MYC was slightly more effective than IR in inhibiting tumor growth, presumably because these treatments did not affect the PKH26- cells. Furthermore, combination of IR with si-c-MYC caused even stronger regression of tumor growth, and relapse was prevented (Fig. 7A, B, Supplementary Figure 5A). Thus, these observations indicate that c-MYC is important for PKH26+ cell formation and radioresistance in vivo.

To determine the basis for why combinatorial therapy of IR with si-c-MYC was more effective than IR alone, we examined the populations of cells from tumors. After treatment, the PKH26+ and CD44+ population were nearly absent from mice subjected to combinatorial therapy, while they were easily observed in tumors from mice treated with IR alone (Fig 7C). Furthermore, tumors treated with IR and
si-c-MYC showed reduced levels of c-MYC, CHK1/2, and pCHK1/2 as compared to untreated tumors (Fig 7D). We also analyzed Ki67 and Brdu expression by IHC in tumor tissues obtained from the four treatment groups. Ki-67 and Brdu positive cells decreased in the tissues from the combination group of IR with si-c-MYC (Supplementary Figure 5B). To further verify the pathological correlation between c-MYC, CHK1/2 and CD44 in nasopharyngeal carcinoma, we performed a correlation analysis of c-MYC, CHK1/2 and CD44 protein expression levels using immunohistochemistry, respectively, in primary nasopharyngeal carcinoma (NPC) tissue consisting of 62 primary nasopharyngeal carcinoma samples (Fig. 7E, F). Consistently, high-expression of c-MYC positively correlated with increased CHK1/2 and CD44 expression levels. Expression of c-MYC and CHK1/2 also positively correlated with poor differentiation in high grade tumors (Fig. 7G). These data suggest expression of c-MYC could lead to up-regulation of CHK1/2 and activation of stemness signature that contribute to nasopharyngeal carcinoma progression, and c-MYC-CHK1/2 pathway is essential to maintain the formation of and radioresistance of PKH26+ cells.

**Discussion**

Radiotherapy is the most commonly applied treatment for NPC, but tumor recurrence is essentially universal due to marked radioresistance. Cancer stem cells play an important role in radioresistance and tumor repopulation through preferential checkpoint response and DNA repair. Thus, the targeting of the checkpoint response in cancer stem cells may overcome tumor radioresistance. PKH26 is a fluorescent
membrane dye used in combination with other cell surface antibodies for flow cytometric sorting of stem cells (32, 33). PKH26 label retention/quenching characteristics were used in a recent study by Dembinski et al. to identify a subpopulation of stem cell-like, slow cycling tumor cells in pancreatic adenocarcinoma (34). These cells were resistant to chemotherapy and exhibited the highest self-renewal potential (35). It has been shown that this phenomenon is caused by radiotherapy or chemotherapy enrichment of CSCs (36). In the present study, we have discovered and identified a rare population of PKH26+ cells in the NPC cell population that exhibit a delay of cell cycle progression and are enriched for cancer stem cells that are resistant to radiation in comparison to matched PKH26- cells in vitro and in vivo.

Ionizing radiation (IR) leads to cell death by production of irreparable DNA double-strand breaks (DSBs) (37). A hallmark of DNA DSB recognition and repair is histone H2AX phosphorylation (γ-H2AX), a marker of DNA damage (38). Cells commonly respond to DNA-damaging agents by activating cell cycle checkpoints, and cell cycle regulation is perhaps the most important determinant of ionizing radiation sensitivity. Our studies demonstrate that overexpression of c-MYC in NPCs reduces the expression of γ-H2AX, which may indicate that c-MYC prevents DNA damage by IR in NPCs. Also, the current report demonstrates that non-irradiated CSCs are predominantly in the G0 phase of the cell cycle, while radiation mobilizes CSCs from a quiescent to a proliferative state (G2 phase) (18, 39). ATM is the most proximal signal transducer initiating cell cycle changes after DNA damage induced by
ionizing radiation (40) (Supplementary Fig 1C). Studies in glioblastoma have demonstrated that CD133+ glioma cells promote radioresistance by activation of the CHK1 and CHK2 checkpoint kinases, thus contributing to DNA damage repair (11). In our study, after PKH26+ cells are exposed to radiation, DNA damage activates the phosphorylation of CHK1/CHK2. Thus, CHK1 and CHK2 also play an important role in DNA damage repair in PKH26+ in NPC.

c-MYC is a proto-oncogene that encodes a transcription factor that regulates cell proliferation, growth, and apoptosis (41). OCT3/4, SOX2, c-MYC, and KLF4 are four transcription factors that have been shown to successfully induce pluripotent stem cells (iPS) from human fibroblasts, thus extending the study of MYC in the stem cell field (42). Overexpression of c-MYC is detected in many human tumor cells, indicating that this factor is associated with cell proliferation. Cells expressing MYC override an arrest imposed by physiological levels of the CDK inhibitor P21, which mediates cell cycle arrest by p53 (43). At a molecular level, c-MYC prevents growth arrest and drives cell cycle progression through the transcriptional regulation of MYC target genes. Marhin suggested that MYC suppresses induction of the DNA damage inducible GADD45 gene (44). c-MYC functions to regulate the cell cycle through activation of cell cycle promoting genes, such as CDC25A, CDK4, and cyclins D2, E, and A. It also exerts inhibitory effects on expression of other cell cycle genes, such as gas1, p15, and p21 (45), demonstrated that, compared with normal nasopharyngeal cells, let-7 was downregulated in NPC cells, and the expression of c-MYC inhibits cell proliferation. Transcription factors E2F and MYC are also involved in controlling
cell proliferation and apoptosis through induction of miRNA-17-5p and mir-20a. These findings expand the known classes of transcripts within the c-MYC target gene network (13). Our results demonstrate that c-MYC is an essential factor in PKH26+ cells to maintain cancer stemness and DNA damage repair capacity in response to IR. As previously mentioned, c-MYC directly regulates CHK1 and CHK2 by interaction with their 5' promoter regions. c-MYC expression was increased at both the protein and mRNA levels with increasing doses of irradiation. Additionally, phosphorylation of CHK1 and CHK2 increased, and overexpression of c-MYC reduced the extent of DNA damage after IR. Furthermore, c-MYC altered radioresistance in PKH26+ cells through regulation of CHK1 and CHK2 expression levels (46). Understanding the molecular mechanisms underlying the PKH26+ subpopulation will be crucial for developing a therapeutic approach to overcome NPC radioresistance.

Changes in DNA damage repair influence cell survival, and cell cycle checkpoint controls are the key to controlling the DNA damage repair response. This effect has been confirmed in CSCs. The results of this study demonstrate that the PKH26+ subpopulation of NPC cells exhibits characteristics of CSCs, including strong radioresistance. Radioresistance of PKH26 cells was found to be due to promotion of DNA damage repair by activation of CHK1/2 proteins. However, both in PKH26+ cells and in the NPC cell lines CNE1 and CNE2, CHK1/2 and c-MYC expression were found to be important in the response to radiation. In order to explore the interaction between c-MYC and CHK1/2, bioinformatic and molecular biological investigations of the CHK1/2 promoter regions were conducted. Previous study
suggested that Chk1 inhibitors should be evaluated as potential drugs against Myc-driven malignancies such as certain B-cell lymphoma/leukemia, neuroblastoma, and some breast and lung cancers (47). Our results confirmed that c-MYC exerts a direct regulatory effect on CHK1/2. Therefore, it was proposed that overexpression of c-MYC in the PKH26+ cell subpopulation directs positive regulation of CHK1/2, leading to increased DNA damage repair capacity in these cells. Moreover, an increased proportion of SP cells and tumorsphere formation capacity were observed in NPC cell lines expressing CD44, a CSC marker. It can be speculated that overexpression of c-MYC not only increases the stemness of these cells, but also increases the DNA repair capacity through overexpression of CHK1/2, thereby increasing the radiation resistance of tumor cells. This suggests that the c-MYC-CHK1/2 pathway is a strong causal factor in DNA repair and a driving force in maintaining the stemness in PKH26+ cells. Possibly, targeting the c-MYC-CHK1/2 pathway in PKH26+ cancer cells can overcome NPC radioresistance in vitro and in vivo, which may provide a therapeutic advantage to reduce recurrence.

The findings presented here have allowed us to reach a number of important conclusions. First, we have identified a rare population of PKH26+ cells in the NPC cell population that have many stem cell properties, including cell cycle arrest, unlimited proliferation potential, self-renewal, resistance to radiation, and strong tumor formation ability in vivo. These findings help support the cancer stem cell hypothesis and tumor maintenance after therapy. Second, we demonstrate that PKH26+ cancer cells contribute to radioresistance and tumor repopulation through
preferential DNA damage response. Third, we identified that c-MYC binds the CHK1/2 promoters to regulate the DNA damage checkpoint response and radioresistance of NPCs. Moreover, inhibition of c-MYC or CHK1/2 could overcome NPC radioresistance in vitro and in vivo. Thus, therapies targeting the c-MYC-CHK1/2 pathway in preclinical and clinical development may provide novel therapeutics to improve the treatment outcome of radiation treatment in NPC.

Legend to Figures

Figure 1. CNE2 cells were incubated and sorted with the PKH26 reagent.

A, Image of labeled CNE1 and CNE2 cells after overnight and 30 days of culture under transmitted light and fluorescent light (Magnification, 20×)

B, Purity of the freshly sorted PKH26+ cell populations was analyzed by FACS staining of PKH26+ cells cultured for approximately 4 weeks and used for subsequent experiments. (Data represent the mean ± standard deviation (SD); N = 3)

C, PKH26- and PKH26+ cells were analyzed by FACS to determine the percentage of cells in the indicated cell cycle phase. Data are based on three independent experiments performed in duplicate. Error bars represent standard deviation from the mean.

D, Western blot demonstrating reduced cyclin D1 and cyclin A in PKH26- cells and increased cyclin B in PKH26+ cells derived from NPC cell lines. GAPDH served as a loading control.

Figure 2. PKH26+ cells represent an enriched cancer stem cell-like
subpopulation and correlate with radioresistance

A, Sorting by flow cytometry and detection of PKH- and PKH+ cells containing SP cells. The percentages of SP cells following preincubation of CNE1 and CNE2 cells with verapamil to block the ATP transporter are indicated. (Data represent the mean±standard deviation (SD); N = 3, * P<0.000, **P=0.000).

B, PKH26- and PKH26+ cell lysates were analyzed by Western blotting with anti-ABCG-2 and anti-CD44. GAPDH served as a loading control.

C, Colony formation assay for PKH26+ and PKH26- cells using irradiated (8 Gy). These cells formed fewer colonies (right panel) compared with the untreated control cells.

D, Dose-survival curves of irradiated PKH26- and PKH26+ cells (range, 0-10 Gy).

E, Representative images of irradiated (8Gy) PKH26- and PKH26+ cells were seeded in tumorsphere culture medium at 1000 cells/well for 7 days. Data shown represent the average sphere count from a representative experiment performed in triplicate wells. Data represent the mean±standard deviation (SD); n= 3, *P < 0.05,**P < 0.05

F, The indicated amounts of PKH- and PKH+ cells from CNE2 cells were transplanted into 21-day old nude mice.

G, Immunohistochemical detection of ki-67 expression in PKH+ and PKH- cells.

Figure 3. Reduced c-Myc expression in NPC cells after irradiation-induced DNA damage

A, Western blot detection of c-MYC , pCHK1, CHK1, pCHK2, and CHK2 in
PKH26+ and PKH26- cells sorted from CNE1 and CNE2 cells assessed 24 h after irradiation (8 Gy).

B, Immunofluorescence staining reveals expression of pCHK1, pCHK2, and c-MYC in irradiated (8Gy) CNE1 and CNE2 cells compared with untreated controls (Magnification, 200×).

C, Overexpression of c-MYC by transient transfection in CNE1 and CNE2 lines. c-MYC and γ-H2AX were analyzed by Western bloting.

D, Top: The presence of DNA damage was assessed in irradiated (8 Gy) CNE1 and CNE2 cells by single-cell gel electrophoresis assays under alkaline conditions (alkaline comet assay). Bottom: Quantification of the percentages of CNE1 and CNE2 cells with comet tails following irradiation at different doses.

E, EdU incorporation by CNE1 and CNE2 cells after 8 Gy IR treatment for 24 hour. Chart shows EdU cell staining compared with Hoechst 33342 stained nuclei (±SE). A minimum of 500 cells was counted for each treatment (Magnification, 100×, with a 20 msec exposure time for image acquisition).

Figure 4. c-MYC transcriptionally activates CHK1 and CHK2

A, Schematic representation of the promoter regions of CHK1 and CHK2 with the putative c-MYC response elements and the structure of wild-type (WT-Luc) and c-MYC-RE-mutant (Mut-Luc) luciferase reporters driven by the promoters.

B, ChIP assay using antibodies against c-MYC on NPC cells. Levels of the c-MYC-binding elements (A–F in A) immunoprecipitated by anti-c-MYC are
expressed as percentage of input chromatin. IgG (immunoglobulin G) was used as a negative control. Data represent the mean±standard deviation (SD); n = 3, asterisk indicates P < 0.05.

C, ChIP assay using antibodies against c-MYC in CNE2 cells with knockdown of c-Myc by shRNA. The levels of c-MYC-binding elements C and E bound by c-MYC. Data represent the mean±standard deviation (SD); n = 3).

D, The fold change of luciferase activity (see b for constructs) driven by wild-type and mutant c-MYC response elements under c-MYC treatment (n = 5, asterisk indicates P < 0.05)

E, Western blot detection of c-MYC, CHK1, and CHK2 in vector control and c-MYC expressing NPC cells.

F, mRNA expression levels in vector control and c-MYC expressing CNE1 and CNE2 cells expressing the indicated constructs. Data represent the mean±standard deviation (SD); n = 5.

**Figure 5. c-MYC expression in PKH26 + cells.**

A, CNE1 and CNE2 cells after treated with empty vector and two different shRNAs targeting c-MYC were lysated and analyzed by Western blotting with anti-c-MYC. GAPDH served as a loading control.

B, Flow cytometric profiles of SP cells within the PKH26- and PKH26+ CNE2 cell populations after treatment of c-MYC or sh-c-MYC. The percentages of SP cells are indicated. Data represent the mean±standard deviation (SD); n = 3.

C, Images showing tumor sphere formation in PKH26- and PKH26+ NPC cells
expressing the indicated constructs (magnification, 20×). Columns in chart show number of spheres counted in 10 fields for each group.* P < 0.05

D, Alkaline comet assay of vector control and c-MYC infected irradiated (8 Gy) PKH26- and PKH26+ NPC CNE2 cells. Quantification of the percentages of cells with comet tails following irradiation at different doses.

E, EdU incorporation by irradiated (8 Gy) PKH26- and PKH26+ NPC cells at 24 h. Columns in chart show EdU cell staining compared with Hoechst 33342 stained nuclei (Data represent the mean±standard deviation (SD); n=3). (Magnification, 40×, with a 20 msec exposure time for image acquisition).

F, Western blot detection of γ-H2AX in whole cell lysates of irradiated (8 Gy) NPC cells collected after 24 h.

**Figure 6. Increased radiosensitivity in PKH26+ cells following inhibition of c-MYC or upregulation of CHK1 and CHK2**

A, Western blot analysis of c-MYC, CHK1, and CHK2 expression in CNE1 and CNE2 cells.

B, Right: Alkaline comet assay of vector control and shCHK1+shCHK2 infected irradiated (8 Gy) PKH26+ NPC CNE2 cells. Left: Quantification of the percentages of cells with comet tails following irradiation at different doses. (Data represent the mean±standard deviation (SD); n = 3; *P < 0.05)

C, Clonogenic survival of treated cells seeded at different densities (*P<0.001, **P<0.001)

D, PKH26- and PKH26+ cells were treated or not with 8 Gy of IR, sh-c-MYC, or a
combination of both in CNE1 and CNE2 cells. Representative images of colony formation are shown.

E, PKH26+ cells sorted from CNE1 and CNE2 cell lines were infected with empty vector control, sh-c-MYC, and sh-c-MYC+CHK1/CHK2 plated at equal density and treated with IR. Giemsa stained and photographed.

F, Western blot detection of γ-H2AX in whole cell lysates of irradiated (8 Gy) PKH26+ cells collected after 24 h.

**Figure 7. Combination of sic-MYC and IR Prevents Tumor Relapse in vivo**

A, Tumor incidence in nude mice injected with PKH26+ from CNE2 cells. Tumor volume (mm³) in xenografts treated with Control (siGPF treated but without IR) IR (8Gy), IR (2×4Gy), si-c-MYC, and combinations at days 10, 24, 38, 52.

B, The weights of tumors from xenografts after the indicated treatment of 55 days, Data represent the mean±standard deviation (SD); n=4.

C, Percentage of PKH26+ and CD44+ cells derived from NPC tumors treated with IR or IR combined with si-c-Myc.

D, Western blot analysis of c-MYC, CHK1, CHK2, pCHK1, and pCHK2 expression in the tumor tissue after the indicated treatment. GAPDH used as normalization control.

E, Representative cases from 62 primary nasopharyngeal carcinoma specimens were analysed by immunohistochemical staining (c-MYC, CHK1, CHK2 and CD44). (Magnification, 40X).

F, Expression level of c-MYC, CHK1 and CHK2 in low grade tumours (grade I & II)
versus high grade tumours (grade III \( n = 62, P < 0.001 \)). (-): negative, (+): positive.

G, Expression level of c-MYC versus CHK1, c-MYC versus CHK2 and c-MYC versus CD44 (\( n = 62, P < 0.002 \)).

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**References**


Figure 1

A

Light  Pkh26+  Merge

1 Days

30 Days

B

Week 1  Week 2  Week 4

CNE1

CNE2

C

PKH26-

PKH26+

% Of Cells

G1  S  G2/M

G1  S  G2/M

D

Cyclin D1

Cyclin A

Cyclin B

GAPDH

CNE1

PKH26-

PKH26+

CNE2

PKH26-

PKH26+
Figure 3

A

<table>
<thead>
<tr>
<th></th>
<th>CNE1</th>
<th></th>
<th>CNE2</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR(8Gy)</td>
<td>PKH26-</td>
<td>+</td>
<td>PKH26+</td>
</tr>
<tr>
<td>c-MYC</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pCHK1(S345)</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</tbody>
</table>

B

IR(8Gy)

PKH26-

FITC Merge

c-MYC

pCHK1

pCHK2

PKH26+

FITC Merge

c-MYC

pCHK1

pCHK2

CNE1

CNE2

C

8Gy vector c-MYC

c-MYC

γ-H2AX

GAPDH

D

CNE1 vector c-MYC

0Gy

8Gy

CNE2 vector c-MYC

E

CNE1 vector c-MYC

Average Number Of Positive Cells (%)

P=0.000

CNE2 vector c-MYC

Average Number Of Positive Cells (%)

P=0.000

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 4

A

B

C

D

E

F

arrowed

Normalized FL/RL

vector

CHK1

WT

Mut

vector

c-MYC

CHK1

c-MYC

CHK2

GAPDH

vector

c-MYC

CHK1

CNE1

CNE2

Fold Change of mRNA

vector

CNE1 CNE2

CHK1

CNE1 CNE2

CHK2

CNE1 CNE2

ATM
Figure 7
MYC regulation of CHK1 and CHK2 promotes radioresistance in a stem cell-like population of nasopharyngeal carcinoma cells

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